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ORIGINAL ARTICLE

Evaluation of the Antifungal Effect of Fluconazole and Ivermectin against *Candida albicans*

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C. albicans. Fluconazole. Ivermectin. synergistic effect

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ABSTRACT

Background: *Ivermectin is (macrocyclic lactones) which are products or chemical derivatives of soil microorganisms belonging to the fungus Streptomyces avermitilis.* ***Objective****: The aim of the study is to evaluate the antifungal efficacy of Fluconazole and Ivermectin individually and in combination against Fluconazole-resistant Candida albicans isolates in vitro and in an in vivo rat model.* ***Methodology:*** *Twenty-five Fluconazole-resistant C. albicans isolates were tested for synergistic effects of Fluconazole and Ivermectin. Thirty-two male rats with/without infections will received treatments and fungal burden and survival was assessed. The antifungal efficacy was determined by measuring the zone of inhibition in a well diffusion experiment.* ***Results:*** *The MIC was 3.7 µg for Ivermectin and 11.1 µg for Fluconazole. Antifungal assay of Ivermectin alone (8µg) showed large inhibition zone (15.1 mm) compared to (3.7µg) showed inhibition zone (11.9 mm) and (1 µg) small inhibition zone (8.63mm). Antifungal assay of Ivermectin in combination with Fluconazole (8-11µg) show large inhibition zone (40 mm) compared to (3.7-11µg) less inhibition zone (25.9 mm) and last combination (1-11µg) show small inhibition zone (12.1mm). Animal study of Ivermectin shows the best combination with Fluconazole which showed faster healing time and reduce size of infection from (2-0.2cm) and disappeared redness of skin and return growth of hair is (8-11 µg) of Ivermectin.* ***Conclusion****: Ivermectin showed potent antifungal activity against Fluconazole-resistant C. albicans with MICs ranging from 3.7-100µg/ml. Combining Ivermectin and Fluconazole demonstrated synergistic effects leading to faster treatment response and complete resolution of fungal infection within 21 days in the in vivo model.*

INTRODUCTION

Every year, fungal infections affect the lives of at least 12 million individuals, killing more than 1.5 million of them[1]. Many dangerous fungal pathogen have developed resistance as a result of widespread use of fungicides and prophylactic antifungal therapy, thus the present antifungal armament urgently needs to be expanded[2]. The most well-researched and common human fungal infection is *Candida albicans*. Candida species are fungi that grow as yeasts and are "imperfect," which means they don't seem to have a full sexual cycle[3]. In addition to the budding yeast and pseudo hyphal (elongated yeast) cells found in other Candida species and in the model yeast Saccharomyces cerevisiae, *Candida albicans*, which is believed to be an obligatory diploid, may also generate true filamentous hyphae[4].

The opportunistic pathogen *Candida albicans* lives in the gut, genital tract, urinary tract, and skin as a harmless commensal. It can turn into an opportunistic pathogen in a variety of host conditions, typically involving weakened immune function or an imbalance of the competing bacterial microflora[5]. Azoles are antifungal substances that fall into two categories: Imidazoles and Triazoles[6]. Two nitrogen atoms and a complicated side chain connected to one of the nitrogen atoms make up the five-membered ring structure of Imidazole compounds, which include Ketoconazole, Clotrimazole, and Miconazole[7]. The 14α-sterol demethylase enzyme, which transforms lanosterol into ergosterol by removing the 14α-methyl group from it, is inhibited by the azole chemicals[8].

In 1987, Ivermectin was authorized for use in humans[9]. Head lice, scabies, riverblindness (onchocerciasis), strongyloidiasis, trichuriasis, ascariasis, and lymphatic filariasis are among the infestations that it is used to treat[10]. Ivermectin is a broad-spectrum antiparasitic drug that has been used extensively to treat a variety of parasitic infections in both humans and animals. Ivermectin belongs to the avermectin family of drugs and works by interfering with the nervous system of parasites causing paralysis and death. the primary use of Ivermectin is the treatment of onchocerciasis also known as river blindness a condition caused by the parasitic worm Onchocerca volvulus[11]. It can be taken by mouth or applied to the skin for external infestations[12].

Ivermectin is a macro cyclical lactone ring[13]. It is the outcome of Streptomyces fermentation. Eight closely related avermectin homologues are produced by the Streptomyces bacterium species Avermitilis, of which B1a and B1b make up the majority of the isolated products[14]. Ivermectin, an 80:20 mixture of the two 22,23-dihydroavermectin molecules, is produced by hydrogenating the mixture in a different chemical step[15].

This work expands the breadth of Ivermectin's known pharmacological actions and demonstrates its potential as an anti-mitotic drug. Ivermectin can bind to and stabilize microtubules (i.e., change the tubulin polymerization equilibrium), which can then result in mitotic arrest[16]. Avermectin has been demonstrated to inhibit chitin synthetase and chitin turnover, suggesting that it may be an efficient inhibitor of the enzyme chitinase. It is hypothesized that avermectin can kill vulnerable species by preventing chitin production and turnover at low concentrations[17].

The aim of the study is to evaluate the antifungal efficacy of Fluconazole and Ivermectin individually and in combination against Fluconazole-resistant *Candida albicans* isolates in vitro and in an in vivo rat model.

METHODOLOGY

This prospective case-control study was conducted from April 202٤ to October 202٤. A total number of 25 clinical *candida albicans* isolates resistance to Fluconazole were gathered for the study from Basrah University/Collage of science/Microbiology Department. According to microbiological guidelines, various clinical specimens were taken from patients admitted to Al-Fayhaa Hospital. Identification of isolates were done by Vitek-2 system, which was taken as the gold standard method.

Isolation and identification of *Candida* spp.

Sabouraud dextrose agar (SDA) (Oxoid, UK) was used for inoculation of clinical specimens and incubated at 37 °C for 24-72 h. The colonies were examined microscopically after Gram staining. Identified *Candida* isolates were further categorized to the species level by the standard protocol that includes germ tube test (GTT) and Vitek 2 compact system.

Primary identification of isolates to species level

GTT was used to categorize *Candida* isolates into *C. albicans* and *NAC* spp. It is positive for *C. albicans* and *C. dubliniensis* and negative for other species[18].

Primary identification by chromogenic agar medium

Further species identification to *C. albicans, C. tropicalis, C. krusei*, and other species was done using chromogenic media HiCrome™ *Candida* Differential agar (HiMedia, Mumbai, India) and incubated aerobically at 30°C for 48 hours. The colony color was recorded and interpreted following the manufacturer's instructions.

Confirmation by broth micro dilution

All Isolates were further tested by the broth micro dilution method for their identification and testing of their antifungal susceptibility.

Antifungal susceptibility test

Identified *Candida albicans* isolates were tested for their antifungal susceptibility by agar well diffusion method against one antifungal agent namely: Fluconazole, and dietary supplement silymarin. Broth micro dilution method was used to determine the minimal inhibitory concentration (MIC) for clinically relevant *Candida* spp. using the following antifungal agent; Fluconazole and dietary supplement silymarin[19].

In Vitro Susceptibility of *Candida albicans* to Fluconazole and Ivermectin Combination

Broth Microdilution Assay

The inhibitory effects of Fluconazole alone and in combination with Ivermectin against *Candida albicans* were evaluated using broth microdilution. Stock solutions of Fluconazole (33 mg/mL) and Ivermectin (96 mg/mL) were prepared in DMSO. Serial three-fold dilutions yielded six concentrations of Fluconazole (100, 33.3, 11.1, 3.7, 1.2, and 0.4 µg/mL) and Ivermectin. The minimum inhibitory concentration (MIC) was determined as the lowest concentration inhibiting visible growth[20] with slightly modification.

Agar Well Diffusion Assay

The agar well diffusion method assessed the antifungal activity of Fluconazole and Ivermectin combinations. Mueller-Hinton agar plates were inoculated with *Candida albicans*. Wells received 50 µL of Fluconazole (11 µg/mL) and Ivermectin (1, 3.7, and 8 µg/mL) solutions. After overnight incubation at 37°C, antifungal activity was measured by inhibitory zone diameter[21] with slightly modification.

Animal Study

The study consisted of eight groups (A-H) of rats, each receiving a different treatment regimen. The groups were monitored for 21 days, with wound area measurements and weight recordings taken at regular intervals. Eight groups of rats (n=4) were used. Rats were housed in separate cages at 30% humidity, 22°C, and a 12-hour light-dark cycle. Standard provender and water were provided[22], with slightly modification. Animal handling protocols were approved by Basra University's animal ethics committee.

Induction of cutaneous candidiasis and treatment protocol.

Cutaneous candidiasis was induced in rats by injecting a yeast stock suspension containing 1-5×10^6 *Candida albicans* cells/ml (OK631832) into groups A-H. The wound area was measured using the rule technique (length × width), and rat weights were recorded on days 0, 3, 6, 9, 12, 15, 18, and 21.

Preparation of Skin for Transdermal Application

Rats' back hairs were shaved using a hair removal shaving machine, and a 2 cm^2 region was marked for

application of the formulations. The following day, a Derma roller was used to create micropores, facilitating transdermal delivery of the medication. The rats received a single treatment once daily for 8 days. The control group (positive) received Fluconazole, while the untreated group received no treatment. After 8 days, the responses of the treated groups were compared to those of the control group.

RESULTS

The isolated colonies appear creamy white this represent growth of *Candida albicans*, shown in (Figure 1).

Fig. 1: Morphology of *Candida albicans* on Sabouraud dextrose agar

The isolated colonies appear creamy white and *Candida* differential agar appeared of different colors on the chrome according to the *Candida* species. Light green colonies were identified as *C. albicans.*as shown in (Figure 2).

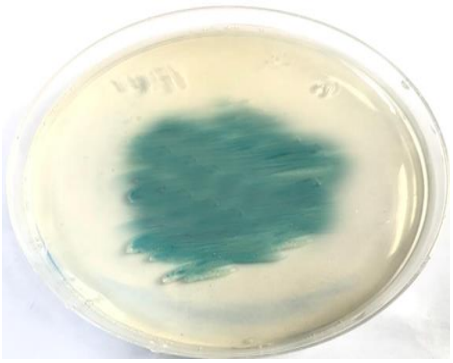

Fig. 2: Growth of *Candida albicans* on chrom agar. HiCrome

The isolates were tested for their ability to form the germ tube to identify them *C. albicans* and other candida species that cannot create the germ tube. The isolates obtained were able to form a germ tube identified as *C. albicans.*as shown in (Figure 3).

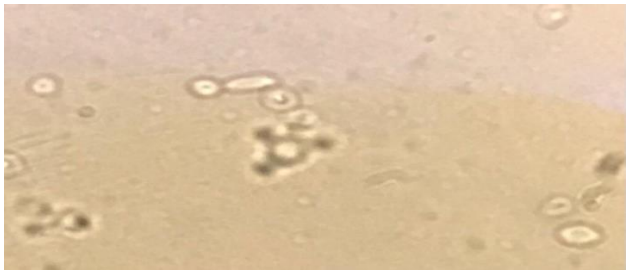

Fig. 3: Germ tube formation of *Candida albicans*

The current study included performing a series of dilutions starting from a concentration of 100µg /ml to a concentration of 0.4µg / ml for the determining the minimum inhibition concentration of drugs on *Candida albicans*. as shown in (Table 1)

Table 1: Serial dilution for detection MIC of Fluconazole and Ivermectin

Substance	Threefold dilution					
	100 µg / ml	33.3 µg/ml	11.1 µg/ml	3.7 µg/ml	1.2 µg/ml	0.4 µg/ml
Ivermectin	+	+	+	+	-	-
Fluconazole	+	+	+	-	-	-

The current study shows that the MIC concentration of Ivermectin against *C. albicans* it was noted that the MIC concentration (3.7µg) was 11.9±0.47, double MIC concentration (8µg) was 15.1±0.45, and half MIC concentration (1µg) was 8.63±0.61 with significant differences between the three concentrations shown in (Table 2) and (Figure 4) below.

Table 2: Activity of Ivermectin alone against *C. albicans*

Concentration	Ivermectin Inhibition Zone mm			
	Frequencies			Mean ± S. D
8	15.2	14.7	15.6	15.1 ± 0.45^{a}
3.7	12.1	11.4	12.3	11.9 ± 0.47^{b}
1	9.3	8.5	8.1	8.63 ± 0.61^{c}
p-value and LSD				< 0.001; 1.03

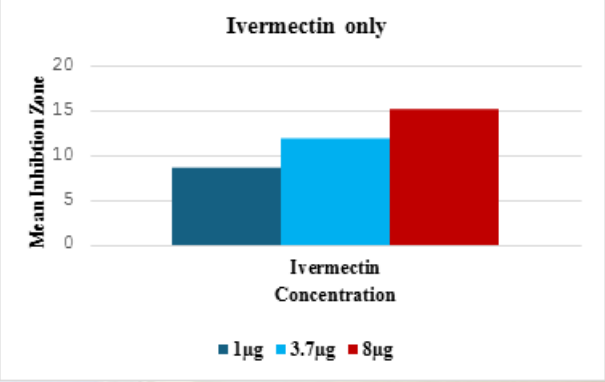

Fig. 4: Activity of Ivermectin alone against *C. albicans*

The results of the current study showed that the effectiveness of Ivermectin against *Candida albicans* increases with increasing concentration when the experiment was repeated three times as shown in (Figure 5).

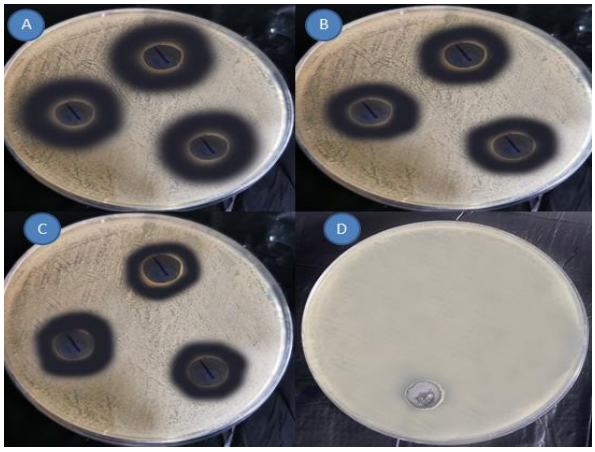

Fig. 5: The agar well method results demonstrate a clear concentration - dependent relationship between Ivermectin and the inhibition zone diameter against *candida albicans*. A- 8µg/ml largest inhibition zone (15.1 mm) indicating highest antifungal activity. B - 3.7 µg/ml moderate inhibition zone (11.9 mm), indicating moderate antifungal activity. C- 1µg/ml, smallest inhibition zone (8.68mm), indicating lowest antifungal activity. compared with D: DMSO control

The current study showed that the activity of Ivermectin combination with Fluconazole against *C. albicans* was increased significantly compared with their activity alone was noted that the 8µg+11µg/ml concentration was 40.0±1.60 and 3.7µg+11µg concentration was 25.9±1.95 and 1µg+11µg concentration was 12.1±1.15 with significant differences between three concentrations, as in the (Table 3) below.

Table 3: Activity of Ivermectin combination against *C. albicans*

Concentration	Ivermectin Inhibition Zone mm			
	Frequencies			Mean ± S. D
8-11	38.2	40.5	41.3	40.0 ± 1.60
3.7-11	28.1	25.4	24.3	25.9 ± 1.95
1-11	12.1	13.3	11	12.1 ± 1.15
p-value and LSD				< 0.001; 3.20

The results of the current study showed that the effectiveness of Ivermectin against *Candida albicans* increases when the antibiotic is mixed with Fluconazole and the effectiveness also increases with an increase in the concentration of Ivermectin as shown in the (Figure 6).

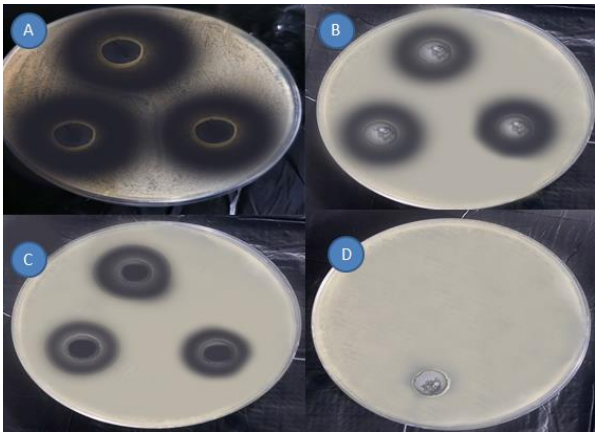

Fig. 6: Activity of Ivermectin combination with Fluconazole against *C. albicans*. A: 1-11µg concentration. B: 3.7-11µg concentration. C: 8-11µg concentration compared with DMSO control (D).

The results of the animal experiment after rats were infected with *Candida albicans* and treated once with Ivermectin alone and combination with Fluconazole showed that the speed of response to treatment was higher with the combination compared with using the Ivermectin alone as shown in the figure 7.

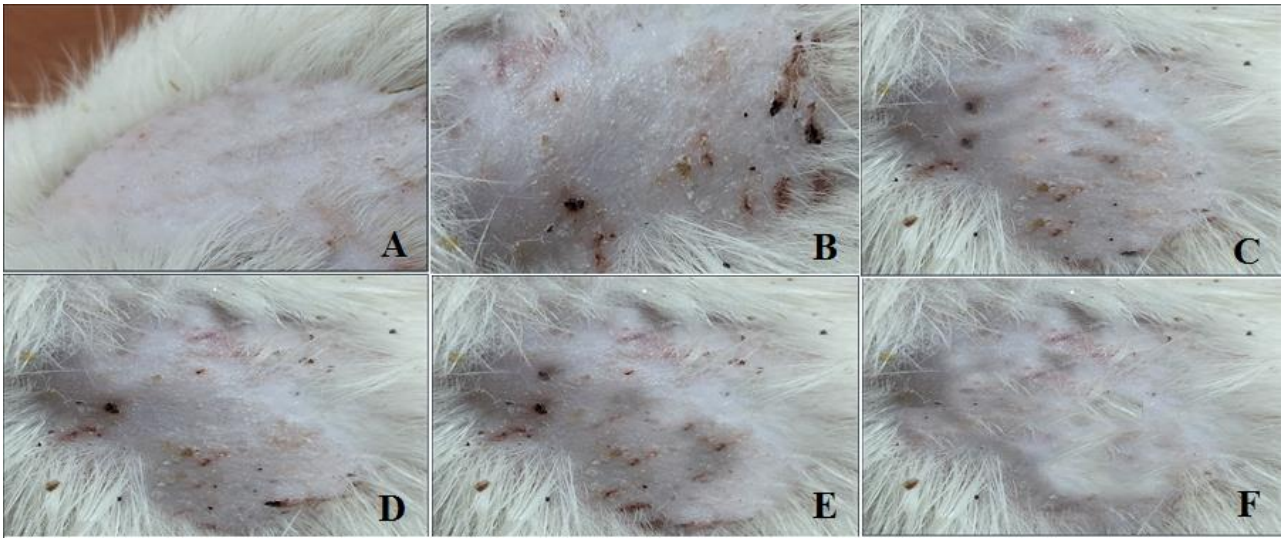

Fig. 7: Mouse skin after fungal infection and treatment A: mice infection with *C. albicans* B: control sever redness, scarring, and hair loss. C: treatment with Ivermectin alone (1µg) mild redness some scaring and partial hair loss, D: treatment with Ivermectin combination 1-11minmal redness reduced scaring and significant hair regrowth. E: treatment with Ivermectin combination 3.7-11al most complete hair re growth minimal scaring and no redness. F: treatment with Ivermectin combination 8-11complet hair re growth, no scaring and healthy skin appearance

The pictures show a progressive healing pattern, with increasing healing percentages corresponding to improved skin appearance. the combination of Ivermectin and Fluconazole resulted in more effective healing compared to individual treatments. Increasing Ivermectin concentration in combination with Fluconazole led to improved healing outcomes.

The rats at which the wounds of the different groups of rats injected with *candida* healing were different significantly from one another**.** The group of rats that received Fluconazole of 11 µg plus Ivermectin of 8 µg concentration reached 100% healing within 15 days of treatment in comparison to those receiving Fluconazole of 11µg plus 1µg Ivermectin reached which 74 % within 15 days of treatment. As a result of the infection, the rats' wounds became acute, suppurative, and vividly red. During the treatment, which lasted from 0 to 15 days, our findings revealed that the groups with varied doses of Ivermectin in conjunction with Fluconazole underwent an exceptionally speedy recovery process. In contrast, the rats treated with Fluconazole only showed a marginal recovery and a substantial weight loss. The rats who were not treated (the control group) did not experience any recovery and instead showed excessive weight loss, ultimately leading to death.as shown in (Figure 8).

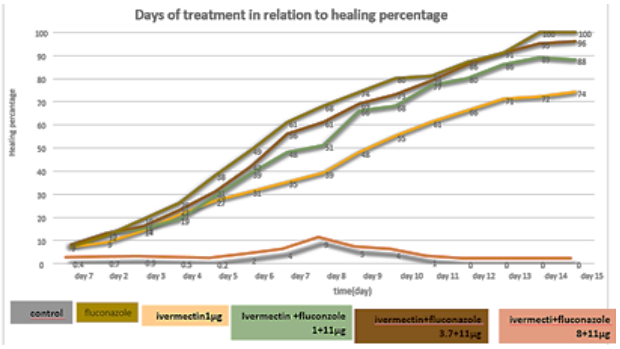

Fig. 8: Healing percentage chart

Skin infections caused by fungal pathogens pose a significant threat to animal health. Rats with skin infections were treated with either Fluconazole only or a combination of Ivermectin and Fluconazole (8+11µg) for 15 days. The combination therapy resulted in a significant reduction in infection size from (2cm to 0.8cm) compared to Fluconazole only (2 cm to 1.8cm). as shown in (Figure 9).

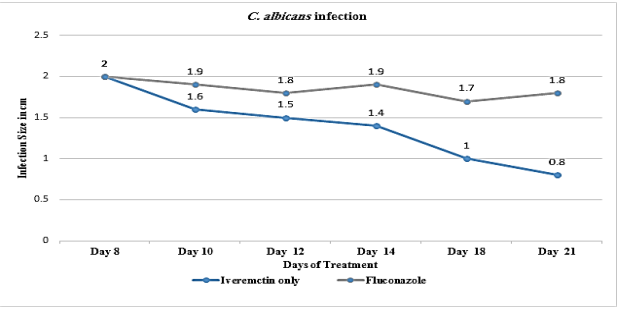

Fig. 9: Association between fungal infection and treatment with Ivermectin combination with fluconzole , and Fluconazole only according to time

DISCUSSION

The ongoing research of novel natural compounds as antimicrobial agents is a result of the emergence of multi-drug resistance in microorganisms and the decrease in the use of antibiotics to treat and control microbial infections[23]. The need for novel broad-spectrum medications to address illnesses brought on by eukaryotic pathogens, such as fungal infections, is well demonstrated by the drug resistance seen with many anti-microbial. Given that these illnesses affect the most vulnerable populations, which are at a disadvantage due to health and socioeconomic circumstances, novel agents should, if at all possible, be simple to create so that their cheap cost can facilitate commercialization. This study demonstrates that the antifungal properties of Ivermectin and Fluconazole against *Candida albicans* not only increase the parent drug's activity but also expand its range of use. All things considered, the identification of these substances with broad-spectrum activity creates new opportunities for the creation of remedies for a number of existing human infections brought on by parasites or fungi, as well as for recently discovered illnesses[24]. The results of these investigations demonstrated that these chemicals have the potential to combat a wide range of illnesses, such as diabetes, cancer, heart disease, osteoporosis, and other human conditions. The majority of these substances are employed as nutraceuticals[25].

Current study was noted the MIC concentration (3.7µg) was 11.9±0.47, double MIC concentration (8µg) was 15.1±0.45, and half MIC concentration (1µg) was 8.63±0.61 to Ivermectin against *C. albicans* with significant differences between three concentrations (Table 3) and the effectiveness of Ivermectin against *Candida albicans* increases with increasing concentration was the experiment repeated three times. in the context of the discovery of new broad-spectrum anti-microbial agents for eukaryotic pathogens including fungi many studies have relied on experiments with treatments specific to parasites to determine their effect on fungi including yeasts. While it is proposed that avermectin can kill susceptible organisms by inhibiting chitin turnover and synthesis at low concentration Ivermectin has been shown to inhibit chitin synthetase and chitin turnover it is conceivable that it might be an effective inhibitor of the enzyme chitinase and that may be the cause in finding significance between concentrations that have effect on *C. albicans* in current study[26].

Studies show that the effectiveness of the Ivermectin combination against *C. albicans* was significantly increased when it was used instead of Ivermectin alone and this is what the current study showed. This improvement in efficacy may be due to the synergistic effect between the components of the formulation or to the improvement of the pharmacological properties of the dosage. The results of the current study are consistent with some previous studies that showed that using the combination of Ivermectin and Fluconazole increases their effectiveness against *Candida albicans* more than using them alone. this synergistic effect may be due to various mechanisms such as influencing fungal cell pathways or improving the pharmaceutical properties of the compounds[27]. However, there are some studies that did not find a clear synergistic effect between Ivermectin and Fluconazole against *Candida albicans* and this may be due to a difference in the concentrations used or study conditions[28,29].

To evaluate skin infection in mice and time after treatment with an antifungal formulation of Ivermectin, a fungal infection with *Candida albicans* 2 cm in diameter was treated with Ivermectin and Fluconazole for 21 days. the fungal infection took 7 days to occur after which the infection was treated and the diameter of the infection was recorded during the eighth day until it completely recovered on the twenty-first day it was found that Ivermectin has antifungal activity against clinical strains of *Candida albicans* and that this effectiveness is increased when used with Fluconazole[30]. Therefore, the present study provides promising evidence of the possibility of enhancing antifungal effectiveness when using this formulation.

In a study on *Malassezia furfur* used sixteen patients were divided in to three group, alcoholic extract of Calvatia craniiformis (1000 mg ,800 mg ,600 mg,400 mg 200 mg, 100 mg), and (0.5%, 1%, 2%) Ivermectin aqueous solution, alcoholic extract of Calvati craniiformis (1000mg) and (2%) Ivermectin significantly inhibit the in vitro growth of *Malassezia furfur* the effect was proportionally associated with concentration, the meantime of clearance for clinical lesions using these agents was shorter than Fluconazole and hence can be used as a novel topical antifungal agent for treatment of PV associated *M. furfur* infections[31].

A study compatible with this study in were used three concentration of Ivermectin 1,3.7,8 µg on *candida albicans* and note that Ivermectin 8 µg on Muller Hinton agar gave large inhibition zones compared to 3.7

µg indicate good activity on *candida albicans* also when make combination between three concentration of Ivermectin 1,3.7,8 µg with 11µg Fluconazole, 8 µg of Ivermectin give best result also in animals study healing time of infected area of mice by *candida albicans* when treated with combination therapy was faster than monotherapy and in combination therapy we used three combination between Ivermectin and fluconzole are 8-11µg, 3.7 -11µg, 1-11µg, the 8- 11µg give faster result so healing time of Ivermectin 3.7 µg was faster from Fluconazole showed that in group A,15/20 (75%) of patients respond to treatment with Ivermectin aqueous solution (2%) meantime for clearance was 23.5 days, in control group, 14/20 (70%) patients respond to Fluconazole (150mg), Meantime for clearance was 41days . This indicate faster healing time and period of therapy shorter with Ivermectin compatible with current result.

CONCLUSION

Ivermectin showed potent antifungal activity against Fluconazole-resistant *C. albicans*, with MICs ranging from 3.7-100 µg/ml. combining Ivermectin and Fluconazole demonstrated synergistic effects leading to faster treatment response and complete resolution of fungal infection within 21 days in the in vivo model. These findings suggest the potential of Ivermectin - Fluconazole combination as a novel therapeutic approach against drug-resistant Candida infections.

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Ethical approval:

All procedures performed on rats were in accordance with the ethical standards of the Ethics Committee of the Institutional Review Board of University of Basrah - College of Pharmacy by (Code: MS-72-2021).

Conflict of interest:

The authors state no conflict of interest for the research, authorship, and/or publication of this article.

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